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CDK4/6 inhibition antagonizes the cytotoxic response to anthracycline therapy

A. Kathleen McClendon,[1,†] Jeffry L. Dean,[1,†] Dayana B. Rivadeneira,[1] Justine E. Yu,[1] Christopher A. Reed,[1] Erhe Gao,[2] John L. Farber,[3] Thomas Force,[2] Walter J. Koch[2] and Erik S. Knudsen[1,*]

[1]Department of Cancer Biology at Kimmel Cancer Center; Thomas Jefferson University; Philadelphia, PA USA; [2]Center for Translational Medicine; Temple University School of Medicine; Philadelphia, PA USA; [3]Department of Pathology, Anatomy and Cell Biology; Thomas Jefferson University; Philadelphia, PA USA

[†]These authors contributed equally to this work.

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Triple-negative breast cancer (TNBC) is an aggressive disease that lacks established markers to direct therapeutic intervention. Thus, these tumors are routinely treated with cytotoxic chemotherapies (e.g., anthracyclines), which can cause severe side effects that impact quality of life. Recent studies indicate that the retinoblastoma tumor suppressor (RB) pathway is an important determinant in TNBC disease progression and therapeutic outcome. Furthermore, new therapeutic agents have been developed that specifically target the RB pathway, potentially positioning RB as a novel molecular marker for directing treatment. The current study evaluates the efficacy of pharmacological CDK4/6 inhibition in combination with the widely used genotoxic agent doxorubicin in the treatment of TNBC. Results demonstrate that in RB-proficient TNBC models, pharmacological CDK4/6 inhibition yields a cooperative cytostatic effect with doxorubicin but ultimately protects RB-proficient cells from doxorubicin-mediated cytotoxicity. In contrast, CDK4/6 inhibition does not alter the therapeutic response of RB-deficient TNBC cells to doxorubicin-mediated cytotoxicity, indicating that the effects of doxorubicin are indeed dependent on RB-mediated cell cycle control. Finally, the ability of CDK4/6 inhibition to protect TNBC cells from doxorubicin-mediated cytotoxicity resulted in recurrent populations of cells specifically in RB-proficient cell models, indicating that CDK4/6 inhibition can preserve cell viability in the presence of genotoxic agents. Combined, these studies suggest that while targeting the RB pathway represents a novel means of treatment in aggressive diseases such as TNBC, there should be a certain degree of caution when considering combination regimens of CDK4/6 inhibitors with genotoxic compounds that rely heavily on cell proliferation for their cytotoxic effects.

Introduction

Triple-negative breast cancers (TNBC) account for 15–20% of all breast cancers yet approximately 50% of breast cancer deaths.[1,2] This poor clinical outcome can be attributed to both the aggressiveness of the disease and limited therapeutic strategies clinically available.[2] In this context, TNBC is ER/PR/Her2-negative and, consequently, unresponsive to both endocrine-based therapies and Her2-targeted agents.[3] As a result, TNBC is often treated with cytotoxic chemotherapy regimens, most of which include anthracyclines (e.g., doxorubicin) that can yield significant side effects that both preclude treatment of patients with existing health conditions and further compromise quality of life.[3,4] Thus, recent studies have been focused on discovering new molecular markers through which to direct novel therapeutic strategies.

Over the last few years, the retinoblastoma tumor suppressor (RB) protein has been associated with disease progression and therapeutic outcome in various cancer types.[5-7] In the context of TNBC, RB pathway deregulation is a frequent occurrence.[8] While this molecular attribute contributes to the aggressive behavior of these tumors, loss of RB function was also shown to be associated with improved response to chemotherapy.[6] Specifically, in a recent study examining microarray data sets of encompassing over 900 breast cancer patient samples, a gene expression signature of RB pathway deregulation was associated with improved response to chemotherapy, including regimens containing anthracyclines, and longer relapse-free survival in ER-negative disease.[6] This sensitivity is thought to be the result of a predilection toward cell death associated with bypass of RB-mediated cell cycle checkpoints that guard against DNA damage.[9,10] Conversely, disease progression was observed in the majority of ER-negative patients receiving the same chemotherapeutic regimens and demonstrating a functional RB pathway.[6] Thus, RB functional status is an important predictor of chemotherapeutic response in TNBC and could potentially represent a marker for which novel targeted therapies could be directed.

Recently, highly specific CDK4/6 inhibitors were developed that represent a viable mechanism for systemic activation of the

*Correspondence to: Erik S. Knudsen; Email: eknudsen@kimmelcancercenter.org
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RB pathway.[11] Preclinical studies from our laboratory and others have demonstrated that CDK4/6 inhibition blocks DNA synthesis by prohibiting cell cycle progression from G_1- to S-phase, resulting in a potent cytostatic effect that is dependent on a functional RB pathway.[12-14] This response has been observed in tumor and non-tumor cell lines as well as tumor xenografts and transgenic mouse models. Importantly, PD-0332991 is currently being tested in the clinic as both a single agent as well as in combination with other targeted agents (e.g., letrozole) and cytotoxic compounds (e.g., paclitaxel, 5-FU). However, there have been no preclinical studies to date that examine the mechanistic impact of PD-0332991 on the cytotoxic response of cancer cells to genotoxic agents such as anthracyclines, which presumably require cell proliferation for efficacy. The current study determines the effect of pharmacological CDK4/6 inhibition on the response of TNBC to anthracycline-based chemotherapy in vitro and in vivo.

Results

CDK4/6 inhibition yields a cooperative cytostatic effect in combination with doxorubicin in TNBC cells but ultimately antagonizes cytotoxicity. While the efficacy of CDK inhibitors and cytotoxic chemotherapy has been individually evaluated in numerous cell models, the additive or antagonistic impacts of these therapies remain unclear. Pharmacological CDK4/6 inhibition via PD-0332991 in RB-proficient breast cancer cells (MDA-MB-231 and Hs578T) results in a dramatic decrease in BrdU incorporation associated with cell cycle arrest in G_1-phase (**Fig. 1A**; **Fig. S1A**) and a corresponding decrease in S-phase-associated factors regulated by RB (cyclin A, topoisomerase IIα) (**Fig. 1B**; **Fig. S1B**). In contrast, doxorubicin treatment does not inhibit BrdU incorporation but leads to accumulation of cells in S-phase and G_2-M of the cell cycle and enhanced levels of S-phase proteins. Importantly, PD-0332991 and doxorubicin co-treatment leads to an intermediate cell cycle distribution with significant inhibition of BrdU incorporation (**Fig. 1A**; **Fig. S1A**) and decreased S-phase protein levels (**Fig. 1B**; **Fig. S1B**), indicating that RB pathway activation is dominant to the effects of doxorubicin in the context of proliferation. Thus, there is a distinct mechanism through which these compounds impinge on cell cycle control, suggesting possible antagonism.

As previously reported,[14] cyclin D1 protein levels accumulate with PD-0332991 treatment (**Fig. 1B**; **Fig. S1B**). Interestingly, doxorubicin leads to degradation of cyclin D1, irrespective of CDK4/6 inhibition, suggesting that the DNA damage response is unimpaired in cells treated with doxorubicin despite inhibition of CDK4/6 activity. This was confirmed by phospho-γH2AX (p-γH2AX) staining, wherein cells treated with doxorubicin harbored a significant increase in p-γH2AX foci irrespective of PD-0332991 treatment (**Fig. 1C**). In contrast, while doxorubicin treatment resulted in significant upregulation of pro-apoptotic factor E2F1 and induction of cleaved PARP, these signaling events were attenuated with PD-0332991 treatment (**Fig. 1D**; **Fig. S1B**). Combined, these data indicate that by enforcing RB pathway activation, there is an enhanced cytostatic response but inhibition of doxorubicin-mediated cell death signaling.

CDK4/6 inhibition does not modify the sensitivity of RB-deficient TNBC to cytotoxic chemotherapy. RB deficiency has been demonstrated to increase the sensitivity of human breast cancer cell lines and tumors to cytotoxic chemotherapy.[8,15,16] While RB deficiency has been shown many times to render cells resistant to the cell cycle effects of PD-0332991, it is possible that CDK4/6 inhibitors could have effects outside of the RB pathway.[7] Thus, to determine the impact of CDK4/6 inhibition on the therapeutic response of RB-deficient TNBC to chemotherapy, we utilized two RB-deficient TNBC cell lines (MDA-MB-468 and MDA-MB-436). As has been previously demonstrated,[12-14] PD-0332991 was completely ineffective at suppressing proliferation in RB-deficient cells (**Fig. 2A**). Importantly, PD-0332991 and doxorubicin co-treatment results in cell cycle profiles and proliferation rates virtually identical to those observed with doxorubicin alone. Additionally, there is no effect of PD-0332991 on either the expression of S-phase-associated target genes (cyclin A, topoisomerase IIα) or doxorubicin-mediated degradation of cyclin D1 (**Fig. 2B**), induction of p-γH2AX (**Fig. 2C**) or apoptotic signaling (**Fig. 2D**). In addition to using TNBC cells lines that are naturally RB-deficient, we performed retroviral knockdown of RB in MDA-MB-231 cells, as has been previously described.[14] Similar to results observed in MDA-MB-468 and MDA-MB-436 cells, co-treatment with PD-0332991 did not alter the cellular response of RB-deficient MDA-MB-231 cells to doxorubicin (**Fig. S2**). Specifically, similar cell cycle profiles as well as levels of proliferation and apoptotic cell populations were observed in response to doxorubicin treatment irrespective of PD-0332991 exposure (**Fig. S2**). Combined, these data demonstrate that pharmacological CDK4/6 inhibition does not alter the acute therapeutic response of RB-deficient TNBC cells to anthracycline-mediated cytotoxicity. Furthermore, these data confirm that the aforementioned antagonism observed in RB-proficient TNBC cells is indeed dependent of RB-mediated cell cycle control.

CDK4/6 inhibition antagonizes doxorubicin-mediated cytotoxicity in vivo in an RB-dependent manner. To examine the impact of CDK4/6 inhibition on in vivo tumor response to doxorubicin, mice harboring MDA-MB-231 xenografts were treated with vehicle, PD-0332991 and/or doxorubicin. Consistent with our cell culture studies, CDK4/6 inhibition resulted in a significant decrease in cell proliferation as determined by Ki67 staining in excised tumor tissue (**Fig. 3A**) as well as decreased BrdU incorporation (**Fig. S2**). Interestingly, doxorubicin alone did not inhibit Ki67 expression but exhibited a cooperative effect with PD-0332991 (**Fig. 3A**). The failure of doxorubicin to inhibit proliferation was not associated with DNA damage burden, as the percent of p-γH2AX-postive tumor cells was not influenced by PD-0332991 (**Fig. 3B**). Histological analyses revealed significant nuclear aberrations in doxorubicin-treated tumor tissues, which were largely absent in tumors co-treated with PD-0332991. To further analyze this phenomenon, phospho-histone H3 (pSer10) staining was performed to examine mitotic progression. Consistent with Ki67 staining, vehicle-treated tumors

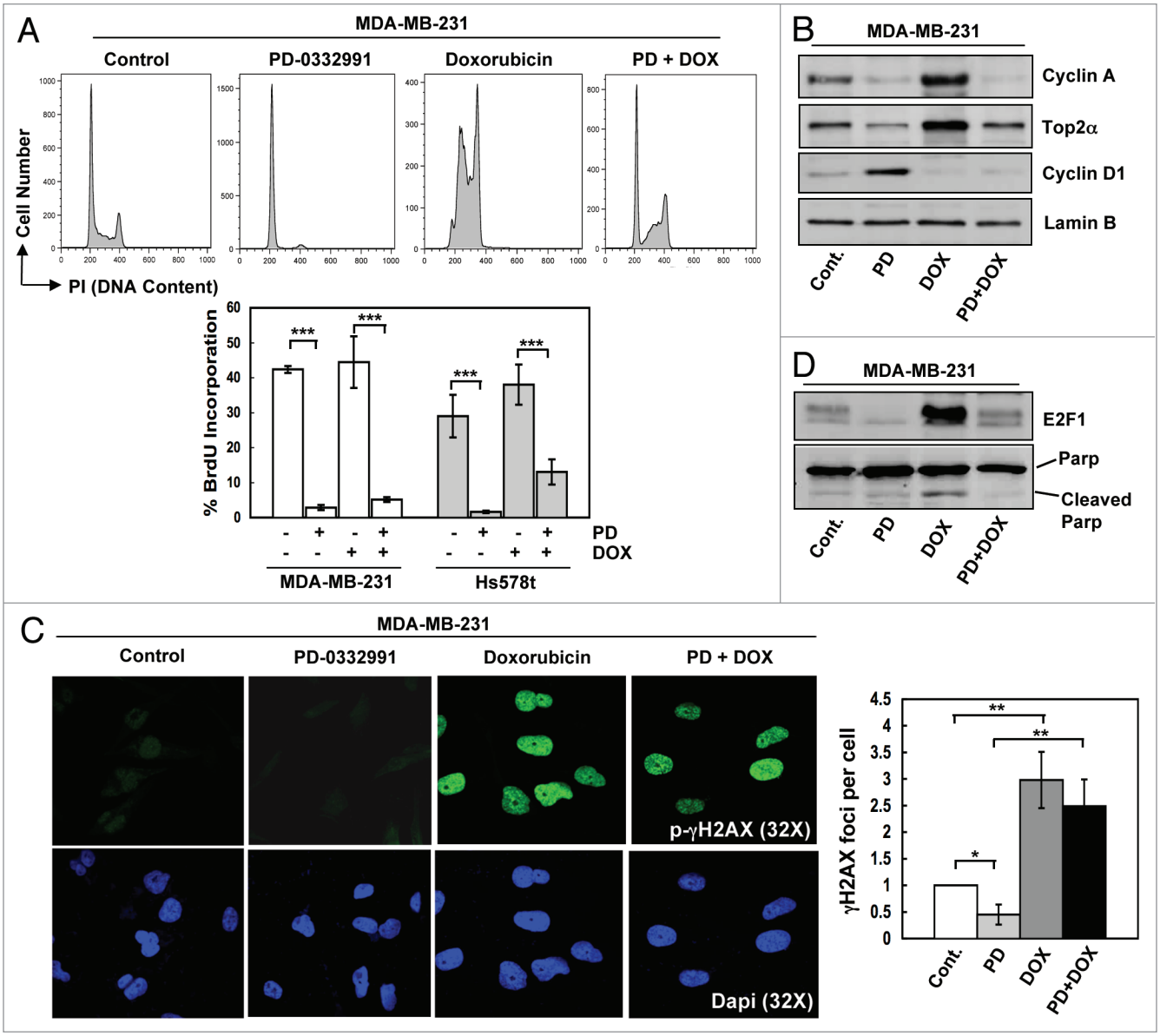

Figure 1. CDK4/6 inhibition yields a cooperative cytostatic effect with chemotherapy but antagonizes cytotoxicity in RB-proficient TNBC cell lines. (A) Representative flow cytometry traces of cells treated with vehicle (Control), PD-0332991 (PD) and/or doxorubicin (DOX) for 24 h are shown, and average percent BrdU incorporation was quantified (***$p < 0.0005$). (B) Cells were treated for 24 h, and total protein lysates were immunoblotted as indicated. (C) Representative images of cells treated for 24 h and stained for p-γH2AX are shown, and average fold increase in p-γH2AX compared with control was quantified (*$p = 0.0074$, **$p < 0.005$). (D) Cells were treated for 24 h, and total protein lysates were immunoblotted for the indicated pro-apoptotic factors.

displayed mitotic figures indicative of normal proliferation, and PD-0332991 treatment resulted in significantly decreased pSer10 staining (**Fig. 3C**). In contrast, doxorubicin treatment resulted in a dramatic increase in pSer10 staining, with a large fraction of cells displaying aberrant mitotic figures and chromosome fragmentation commonly associated with mitotic catastrophe (**Fig. 3C**). This phenotype was completely inhibited by co-treatment with PD-0332991. To directly measure cell death signaling in response to doxorubicin treatment, cleaved caspase-3 staining was performed (**Fig. 3D**). In accordance with our analyses of mitotic fidelity, co-treatment with PD-0332991 effectively inhibited doxorubicin-mediated cell death signaling. Furthermore, PD-0332991 resulted in lower levels of cell death signaling in the absence of doxorubicin treatment as well (**Fig. 3D**). Combined, these studies indicate that doxorubicin and CDK4/6 inhibition yield a cooperative cytostatic response; however, there is antagonism related to apoptotic processes that contribute to the cytotoxicity of chemotherapy.

To confirm the RB dependency of these results in vivo, RB-deficient MDA-MB-231 xenograft tumors were treated with either vehicle, PD-0332991 and/or doxorubicin (**Fig. 4**). In accordance with our in vitro studies, treatment with PD-0332991

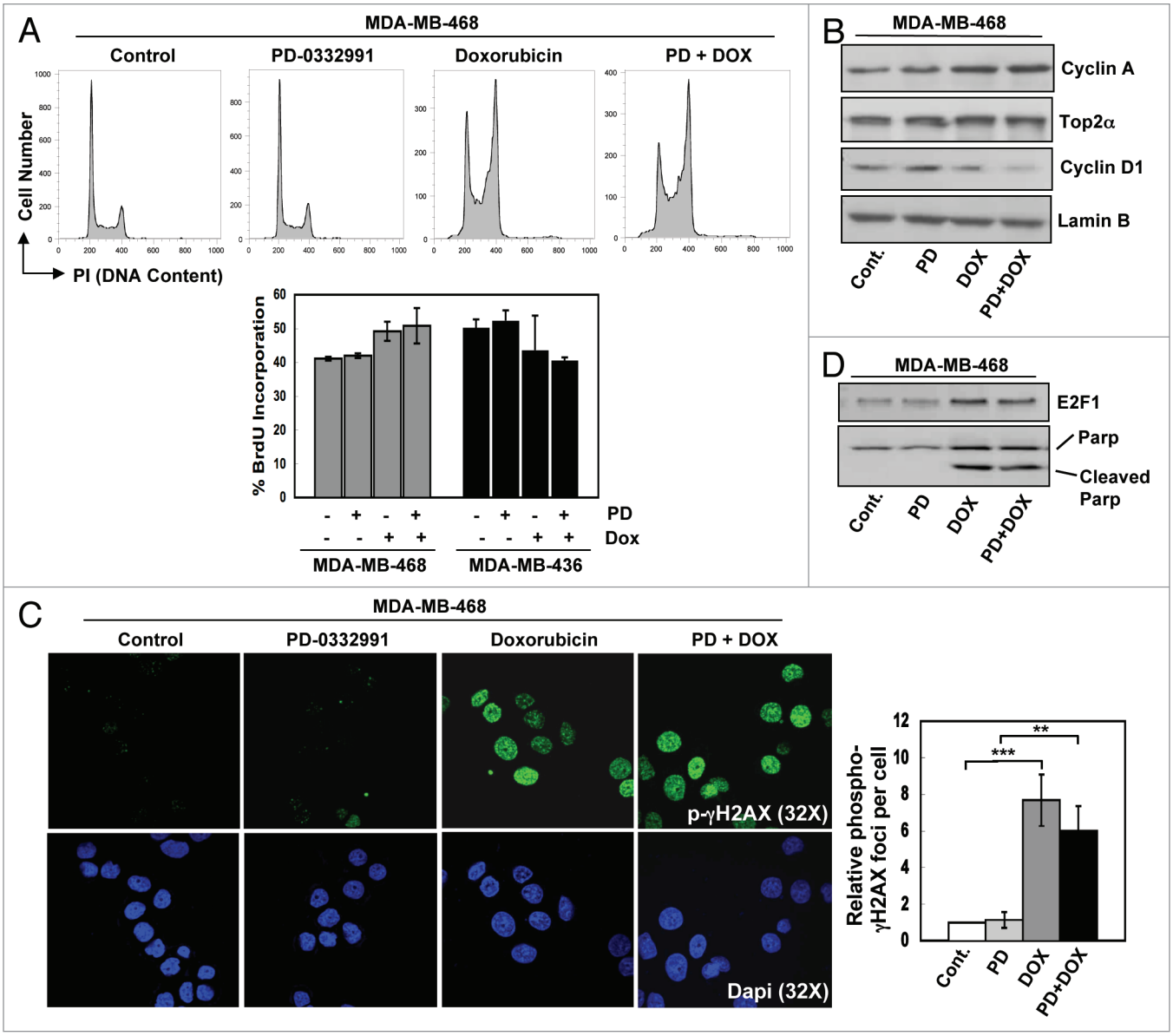

Figure 2. CDK4/6 inhibition does not modify the sensitivity of RB-deficient TNBC cell lines to cytotoxic chemotherapy. (A) Representative flow cytometry traces of cells treated with vehicle (Control), PD-0332991 (PD) and/or doxorubicin (DOX) for 24 h are shown, and average percent BrdU incorporation was quantified. (B) Cells were treated for 24 h, and total protein lysates were immunoblotted for the indicated proteins. (C) Representative images of cells treated for 24 h and stained for p-γH2AX are shown, and average fold increase in p-γH2AX compared with control is shown (**p = 0.0033, ***p < 0.0005). (D) Cells were treated for 24 h, and total protein lysates were immunoblotted for the indicated pro-apoptotic factors.

did not alter the expression levels of Ki67 (proliferation) or p-γH2AX (DNA damage) in comparison to mice treated with vehicle or doxorubicin alone. Furthermore, PD-0332991 treatment did not prevent doxorubicin-induced mitotic catastrophe as observed by pSer10 staining or cell death signaling as observed by cleaved caspase-3 staining (**Fig. 4**). Thus, these data provide in vivo validation that pharmacological CDK4/6 inhibition in combination with anthracycline-based chemotherapy does not alter the therapeutic response of RB-deficient TNBC to the cytotoxic agent.

CDK4/6 inhibition allows for tumor cell outgrowth following doxorubicin treatment. To assess the long-term impact of CDK4/6 inhibition on cell viability following doxorubicin treatment, cellular outgrowth assays were performed. RB-proficient and RB-deficient TNBC cells were treated with PD-0332991 and/or doxorubicin for 24 h, allowed to recover in the absence of drug for the indicated time periods and stained with crystal violet to visualize post-treatment outgrowth (**Fig. 5**). These studies revealed that CDK4/6 inhibition can maintain viability of RB-proficient cells (MDA-MB-231) in the presence of doxorubicin, which then have the capacity to repopulate the culture (**Fig. 5**, top panels). This phenomenon was specifically associated with the presence of a functional RB pathway, as RB-deficient cells (MDA-MB468) were clearly not protected by CDK4/6

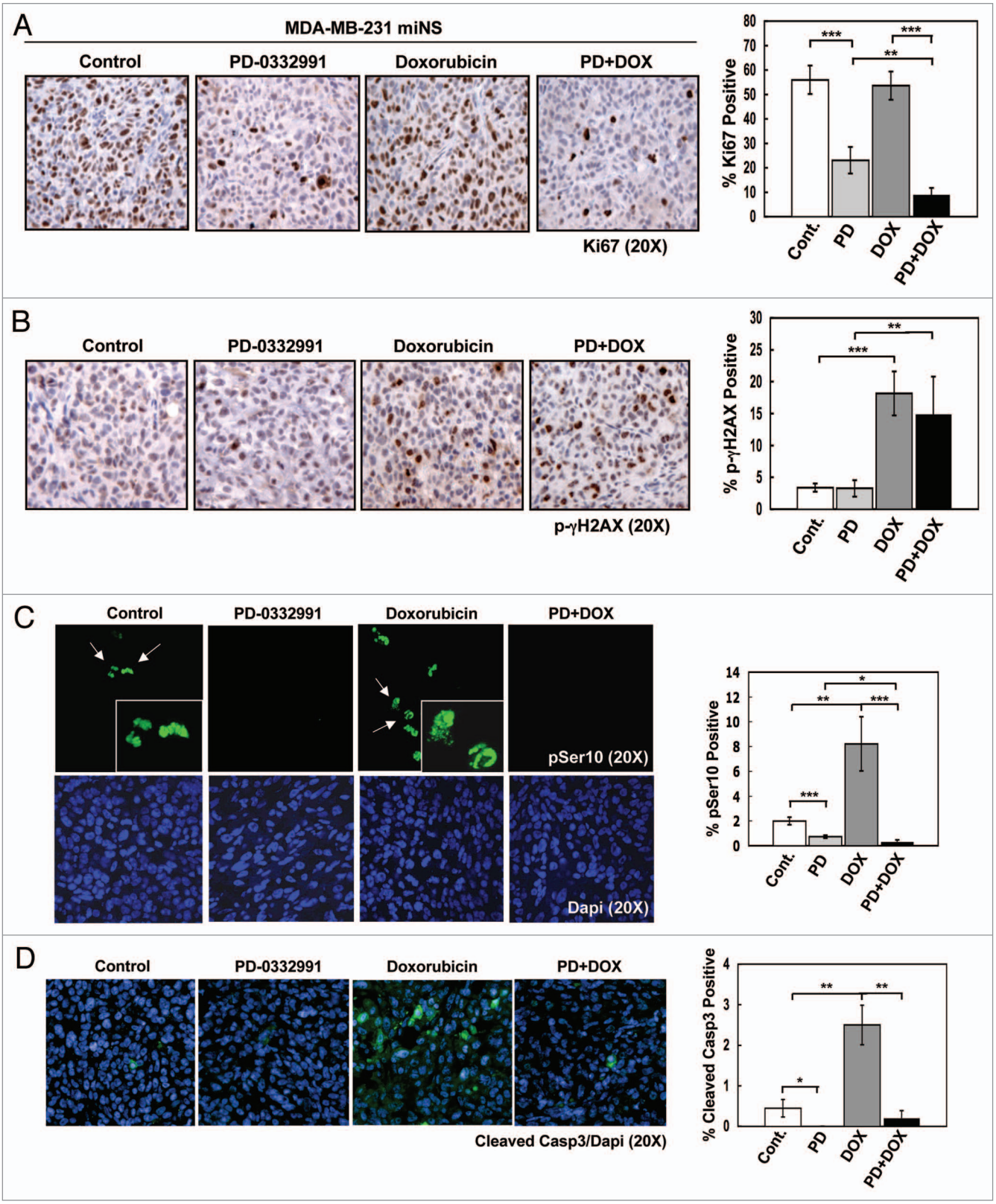

Figure 3. CDK4/6 inhibition antagonizes doxorubicin-mediated cytotoxicity in vivo in an RB-dependent manner. (A) Representative Ki67 staining in xenograft tumors treated with vehicle (Control), PD-0332991 (PD) and/or doxorubicin (DOX) is shown, and average percent Ki67-positive cells was quantified (**p = 0.0065; ***p < 0.0005). (B) Representative p-γH2AX staining in treated xenograft tumors is shown, and average percent p-γH2AX-positive cells was quantified (**p = 0.0032, ***p = 0.0008). (C) Representative phospho-histone H3 (pSer10) staining in treated xenograft tumors is shown, and average percent pSer10-positive cells was quantified (*p = 0.0055, **p = 0.005, ***p < 0.0005). (D) Representative cleaved caspase-3 (Casp3) staining in treated xenograft tumors is shown, and average percent Casp3-positive cells was quantified (*p = 0.023, **p < 0.003).

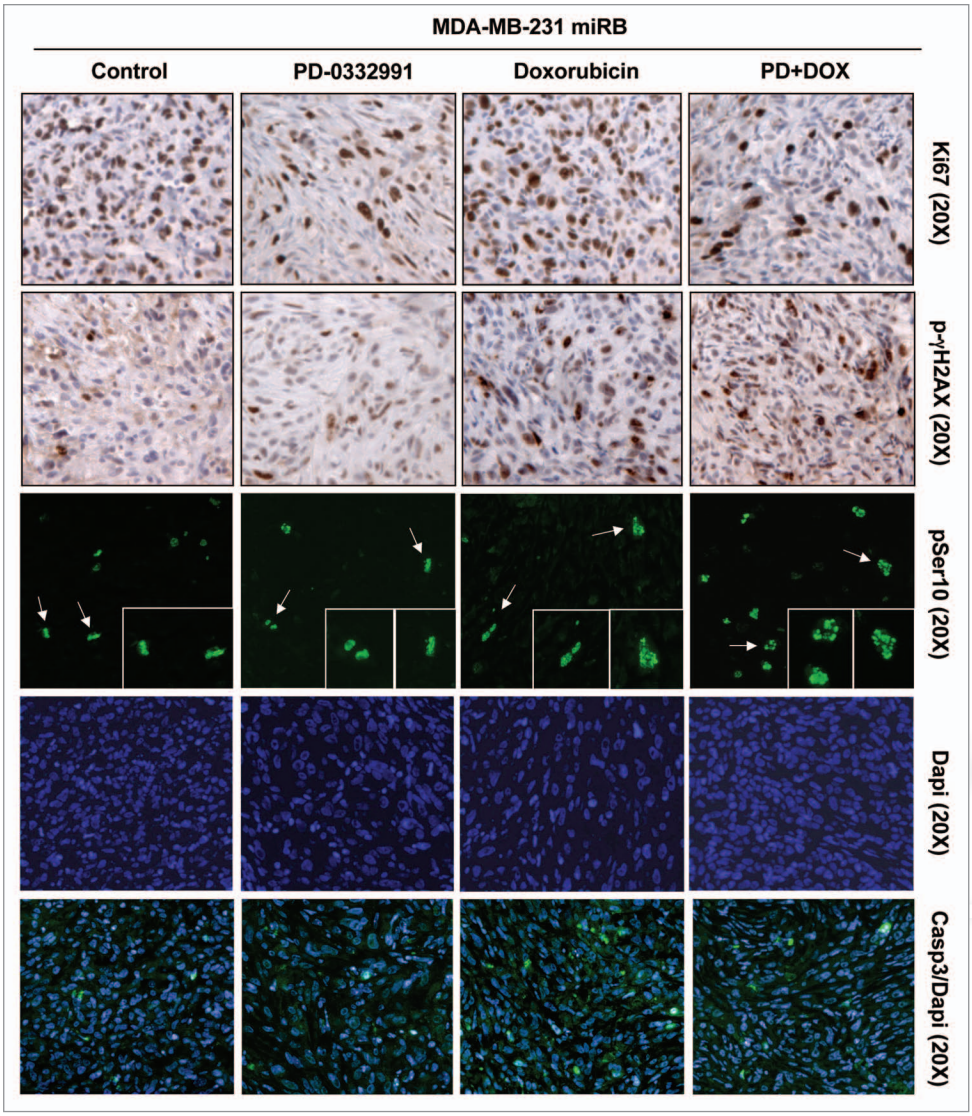

Figure 4. CDK4/6 inhibition does not alter the cytotoxic response of RB-deficient TNBC tumors to doxorubicin. Representative images of Ki67, p-γH2AX, pSer10 and cleaved caspase-3 staining in RB-deficient MDA-MB-231 xenograft tumors treated with vehicle (Control), PD-0332991 (PD) and/or doxorubicin (DOX) are shown. Arrows highlight mitotic figures.

inhibition (**Fig. 5**, bottom panels). The percentage of individual treatment populations displaying outgrowth post-treatment is displayed (**Fig. 5**, right panel). These data demonstrate the potential for CDK4/6 inhibition to ultimately protect tumor cells from anthracylcine-mediated cell death, thus allowing for tumor cell recurrence over time. Taken together, these studies suggest not only mechanistic antagonism between CDK4/6 inhibitors and genotoxic compounds but also the possibility of promoting tumor recurrence by preserving cell viability in the presence of cytotoxic therapies.

Discussion

Efficient application of molecular information to rationally direct therapeutic regimens will be critical for making significant advances in cancer treatment. In the context of TNBC, there are currently no established markers by which to direct targeted therapeutics; thus, virtually all TNBC patients receive cytotoxic chemotherapy regimens.[3] While these chemotherapies can be quite effective at killing tumor cells, they also harbor potentially life-threatening side effects and have a negative impact on quality of life.[3,4] Recent studies have introduced the RB pathway as an important molecular marker for disease progression and therapeutic outcome in cancers, such as TNBC, and thus a viable target for a new class of non-cytotoxic compounds (i.e., CDK4/6 inhibitors).[6] However, the potential for using CDK4/6 inhibitors in the context of currently utilized therapeutic regimens (i.e., cytotoxic chemotherapies) has not been addressed. The current study set out to evaluate the impact of targeting the RB pathway via pharmacological CDK4/6 inhibition in combination with anthracycline-based chemotherapy, a widely used therapeutic modality in TNBC.

In RB-proficient TNBC cell lines and xenograft tumors, there was a cooperative cytostatic effect of pharmacological CDK4/6 inhibition and doxorubicin treatment. Specifically, activation of the RB pathway via CDK4/6 inhibition induces a profound G_1 cell cycle arrest, while doxorubicin treatment yields a G_2/M arrest. Co-treatment yields arrest at multiple points in the cell cycle and corresponding downregulation of proteins required for cell cycle progression via both RB pathway signaling (i.e., cyclin A and topoisomerase IIα) and DNA damage response (i.e., cyclin D1 degradation). Correspondingly, in tumor models, there is a strong suppression of proliferation with the combination treatment. Combined, these data suggest that pharmacological inhibition of CDK4/6 in combination with standard chemotherapies could yield an additive cytostatic effect.

The impact of CDK4/6 inhibition in the context of a cytotoxic response is more complex. While PD-0332991 did not block the induction of p-γH2AX in response to doxorubicin, the cell cycle arrest observed with CDK4/6 inhibition appears to significantly preclude doxorubicin-mediated mitotic catastrophe and cell death signaling in the models herein. Importantly,

combination treatment with PD-0332991 resulted in a recurrent population of cells specifically in RB-proficient cultures that was not observed in cultures treated with doxorubicin alone, indicating that CDK4/6 inhibition can preserve cell viability in the presence of genotoxic agents. Taken together, these findings suggest a degree of antagonism between chemotherapy and CDK4/6 inhibition that will need to be considered in clinical use. While a cytostatic response can be viewed as a positive result in the context of limiting tumor growth, it ultimately serves to inhibit the desired mechanism of cytotoxic agents, which is to kill tumor cells. However, this antagonism could be schedule- and context-specific, such that cycling of PD-0332991 and chemotherapy could be particularly effective in a metronomic setting.[17] Furthermore, aggressive tumor types such as TNBC have been shown to develop resistance to CDK4/6 inhibitors over time[14]; thus, combination regimens with cytotoxic therapies may provide a means to selectively kill those cells that ultimately bypass CDK4/6 inhibition.

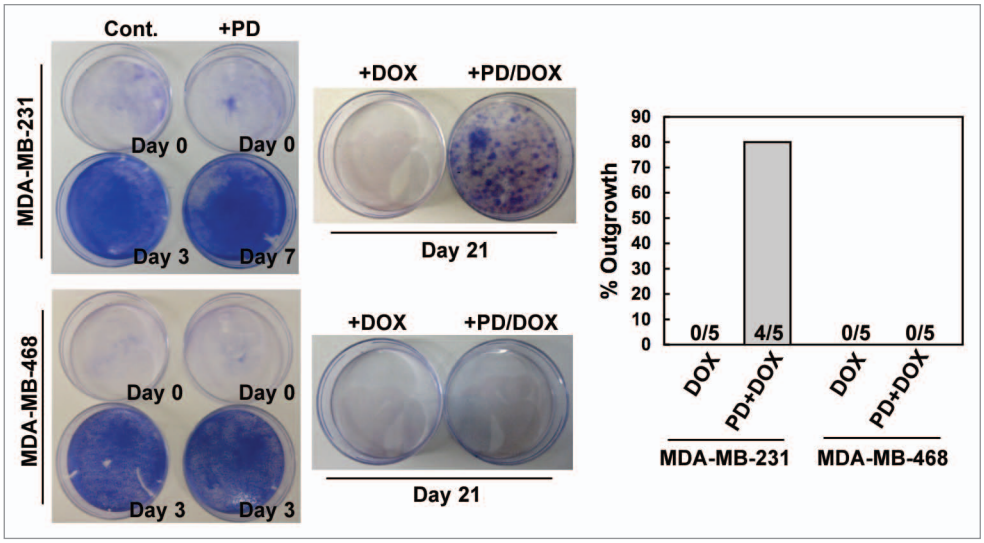

Figure 5. CDK4/6 inhibition in combination with cytotoxic chemotherapy differentially impacts the long-term survival of RB-proficient and RB-deficient cells. RB-proficient (MDA-MB-231) and RB-deficient (MDA-MB-468) cells were treated with vehicle (Control), PD-0332991 (PD) and/or doxorubicin (DOX) for 24 h, allowed to recover in the absence of drug, and stained with crystal violet at the indicated time points post-treatment. Percentages of cell populations displaying clonal outgrowth post-treatment are displayed.

In addition to developing improved treatment regimens to more effectively target cancer cells, there is significant need for therapies that are less toxic to normal tissues. Conventional chemotherapy regimens, most of which include anthracyclines, are associated with significant tissue toxicities that limit their use in the treatment of cancers such as TNBC.[3,4] In this context, the concept of using targeted therapies (e.g., inhibitors of CDKs, p53, MEK, EFG/ErbB, PI3-K) to specifically modulate the cell cycle of normal cells vs. tumor cells was highlighted several years ago, and numerous published studies have supported the potential utility of combining targeted anti-proliferative agents with cytotoxic chemotherapies.[18,19] More recently, Nutlin-3a and Actinomycin D, both pharmacological activators of the p53 tumor suppressor, were shown to protect normal human cells from the toxic effects of mitotic poisons.[20,21] These studies are of particular importance, given that while normal tissues harbor wild-type p53, many tumors are either mutant or deficient for p53 and would be selectively sensitive to cytotoxic compounds. Similarly, a significant fraction of human cancers are RB-deficient.[5] The data presented herein indicate that pharmacological inhibition of CDK4/6 can prevent chemotherapy-mediated DNA damage and cytotoxicity in an RB-dependent manner, suggesting a potential mechanism for protecting normal cells that harbor an intact RB pathway. In this context, a recently published study using mouse models of radiation-induced toxicity indicated that pharmacological CDK4/6 inhibition can modulate hematopoietic toxicity, although the specific mechanism for these effects was not elucidated.[22] Nonetheless, the potential for targeting CDK4/6 in combination with cytotoxic agents as means of selective chemoprotection in normal tissues warrants further study.

Overall, while the new class of CDK4/6 inhibitors provides a promising avenue for therapeutic targeting in cancers such as TNBC that lack established molecular markers for treatment, there should be a certain degree of caution exercised in considering combination regimens with cytotoxic compounds that rely on cell proliferation and accumulation of DNA damage to exert their desired effects. However, by taking advantage of the same mechanism that was shown herein to protect tumor cells from chemotherapy-mediated cytotoxicity, there is the potential for utilizing pharmacological CDK4/6 inhibition as a means for chemoprotection in normal tissues. Thus, assessment of RB status could be effectively used to direct the treatment of cancers while also ameliorating many side effects that negatively influence patient health.

Materials and Methods

Cell culture and treatments. MDA-MB-231, Hs578T, MDA-MB-468 and MDA-MB-436 cell lines were cultured as previously described.[14] miRB and miNS-expressing retrovirus was produced and utilized as previously described.[14] Cells were treated with 500 nM PD-0332991 (Pfizer), 500nM doxorubicin (Bedford Laboratories) or vehicle (dimethyl sulfide, DMSO) (ATCC).

Flow cytometry. Cells were treated with vehicle, PD-0332991 and/or doxorubicin for 24 h, labeled with

BrdU for 1 h, and processed for flow cytometry as previously described.[23] Cell cycle analysis was performed using FlowJo 8.8 software.

Western blot analysis. Lysate preparation and immunoblotting was performed as previously described.[23] Primary antibodies for immunoblotting were Santa Cruz Biotechnology: Cyclin A (C-19), topoisomerase IIα (H-231), Lamin B (M-20); Neomarkers Inc.: Cyclin D1 (Ab-3), E2F1 (Ab-6); Cell Signaling Technology: PARP (9542S).

In vitro phospho-γH2AX immunofluorescence. Cells were plated on coverslips, treated with vehicle, PD-0332991 and/or doxorubicin for 24 h, fixed in 3.7% formaldehyde, and processed as previously described[24] using a monoclonal phospho-γH2AX (p-γH2AX) antibody (Millipore).

Cell outgrowth. Cells were treated with vehicle, PD-0332991 and/or doxorubicin for 24 h, allowed to recover in media lacking drug for the indicated time points, and stained with 1% crystal violet. Assays were performed with five independently treated cell populations.

Tumor xenografts and treatment. Tumors were grown as xenografts in 8-week-old, female athymic nude mice (Harlan Sprague-Dawley, Inc.) by subcutaneous flank injection as previously described.[15] Once tumor volume reached ~100–200mm^3, mice were treated with doxorubicin via single interperitoneal (i.p.) injection (20 mg/kg in 0.9% saline) and/or PD-0332991 daily via oral gavage (150 mg/kg in lactate buffer, pH 4.0) and/or vehicle. Tumor volume was measured throughout the course of treatment, and mice were euthanized after 7 d of treatment. Experiments were conducted in accordance with the NIH Guide for Care and Use of Laboratory Animals and approved by the Thomas Jefferson University Institutional Animal Care and Use Committee.

Histology and immunohistochemistry. Tissues were excised from euthanized mice, and either flash-frozen or fixed in 10% neutral buffered formalin (NBF), paraffin-embedded and cut into 5 μm sections for histology/immunohistochemistry. Mice received a single i.p. injection of 150 mg/kg 5-bromo-2-deoxyuridine (Sigma-Aldrich) in 0.9% saline 1h before sacrifice. Sections were stained with hemotoxylin and eosin (H&E) using standard techniques. Ki67, p-γH2AX, phospho-histone H3 Serine10 (pSer10), and cleaved caspase-3 immunohistochemistry was performed as described.[25] Primary antibodies for immunohistochemistry: Ki67, rabbit polyclonal (Invitrogen Corporation); p-γH2AX, mouse monoclonal (Millipore); pSer10, rabbit polyclonal (Upstate Biotechnology); cleaved caspase-3 (D175), rabbit polyclonal (Cell Signaling). BrdU incorporation was assessed using a Zymed BrdU Staining kit (Invitrogen) according to manufacturer's instructions.

Statistical analysis. Statistical analyses were performed using GraphPad Prism version 4.0 c (GraphPad Prism Software, Inc.). Results were analyzed for statistical significance ($p < 0.05$) using Student t-tests and standard deviation.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Supplemental Material

Supplemental materials may be found here:
www.landesbioscience.com/journals/cc/article/21127

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